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Application of low-pressure system to sustain in vivo bone formation in osteoblast/porous hydroxyapatite composite

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Abstract

Recently, synthetic porous hydroxyapatite (HA) has attracted a great deal of attention as a bone graft substitute in the field of tissue engineering. Combining with bone marrow-derived osteoblasts (BMO), bone formation has been confirmed in vivo and in vitro. However, it is still necessary to obtain more bone formation within porous HA for clinical usage, and, thus, new methods need to be developed. We hypothesized that low pressure during subculture would cause more osteoblastic cells to migrate into the pores of porous HA blocks, resulting in more bone tissue formation in vivo. In the present study, we examined six experimental groups with different pressures from 760 to 10 mm Hg applied to porous HA blocks loaded by bone marrow-derived osteoblasts. For in vivo testing, the 2-week subcultured HA/BMO composites were implanted into subcutaneous sites of syngeneic rats. These implants were harvested at 2, 4 or 8 weeks after implantation. Then, they were prepared for biochemical analysis of alkaline phosphatase (ALP) activity, bone osteocalcin (OCN) content and histological analysis. ALP activity and OCN content in the 100-mm Hg pressure group were highest among the different groups 4 and 8 weeks after implantation ($P < 0.001$). Light microscopy revealed mature bone formation in HA/BMO composite at 4 weeks after implantation. In the scanning electron microscopy (SEM) study, mineralized collagenous extracellular matrix as well as active osteoblasts was observed in HA/BMO composite at 2 weeks after implantation. We concluded that the application of low-pressure system to subculture of bone cells to porous HA blocks is beneficial to increase bone tissue formation in vivo.

Keywords: Hydroxyapatite; Low-pressure system; Osteoblast

1. Introduction

Recently, artificial synthetic porous hydroxyapatites (HA) have attracted attention as a bone graft substitute in the field of tissue engineering because of their high biocompatibility, similar porous morphology to cancellous bone, and good osteoinductivity. Ohgushi et al. [1] showed that the inner surface of porous hydroxyapatite is able to support osteogenic differentiation of marrow stromal stem cells. Yoshikawa et al. [2,3] reported that bone formation in porous hydroxyapatite with bone marrow-derived osteoblasts (BMO) in vivo and demonstrated the osteogenic potential of cultured bone in porous ceramics even after 1 year implantation. Inoue et al. [4] examined the effects of aging on bone formation in porous hydroxyapatite. They concluded that porous hydroxyapatites have a good potential to be available as bone graft substitutes for clinical use in the near future. Some groups have developed methods to induce more in vivo bone formation in porous hydroxyapatite with osteogenic factors [5,6]. However, there has been few study using physical methods.

In practice, osteoblasts show difficulty in invasion (or insertion) into the inner pores of porous HA blocks soaked in the cell suspension. Usually, the ceramic blocks are statically incubated in the cell suspension for several hours. It was different to make some kinds of porous ceramic

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blocks to sink to the bottom of the culture dish, especially for some synthetic porous hydroxyapatites with high porosity. Some cells in suspension would sink to the bottom of the container earlier than the HA block. In the present study, we examined our hypothesis that low pressure would rapidly cause more osteoblasts to migrate into the inner pores of porous hydroxyapatite, resulting in more bone tissue formation in vivo. Here, we describe our method using a low-pressure system for loading cultured cells into porous material with increased efficiency and discuss the appropriate low-pressure conditions.

2. Materials and methods

2.1. Marrow cell preparation and culture

2.1.1. Primary culture

Experiments were performed in accordance with the guidelines of the Japanese Government for the care and use of laboratory animals.

Osteoblastic primary cells were obtained from the bone shaft of the femora of male Fischer 344, 7-week-old rats, according to the method of Maniatopoulos et al. [6]. The femora were excised aseptically, cleaned of soft tissues, and washed three times, for 5 min each, in 6 ml of standard culture medium. Then, both epiphyses were removed, and the marrow was flushed out with 10 ml of culture medium expelled using a syringe with a 23-gauge needle. The obtained cell suspension was distributed into T-75 culture flasks (Falcon, Franklin, Lincoln Lakes, NJ) with 15 ml of standard medium and incubated at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2. The standard medium consisted of Eagle's minimal essential medium (MEM) containing 15% fetal bovine serum (ICN Biomedical Japan) and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B, Sigma). The medium was changed 24 h later to remove non-adherent cells. The remaining adherent cells left were mainly marrow stromal cells [6]. The medium was replaced with fresh medium every other day.

2.2. Subcultures of BMO cells in porous HA and low-pressure system

2.2.1. Porous HA

The sintered porous HA blocks, whose synthetic procedure was described in another paper (Dong et al., to be published), had a high porosity (77%) and were completely interconnected. Average pore diameter was 500 μm and interconnecting path diameter was 200 μm. Fig. 1 shows an SEM picture of the porous HA microstructure.

2.2.2. Subculture of BMO cells in porous HA

When culture dishes became almost confluent after 10 days, they were treated with 0.1% trypsin for 5 min at 37 °C. The cells were counted and concentrated to 10^6 cells/ml by centrifugation at 900 rpm for 5 min at room temperature. The porous HA ceramic blocks were then soaked in a cell suspension within the culture dish. Then, the dish was put inside the vacuum desiccator at low-pressure system as follows.

2.2.3. Low-pressure system

Our low-pressure system consisted of an Ulvac G-5 oil rotary vacuum pump (Sinku Kiko, Japan), Iuchi vacuum controller VC-100 (Tokyo Rikakikai, Japan), and Iuchi Polycarbonate vacuum desiccator (Tokyo Rikakikai), connected to each other by rubber and silicon tubes. Fig. 2 shows the photograph of low-pressure system.

We followed the manufacturer's protocol for use of the vacuum pump and vacuum controller. We took 100 mm

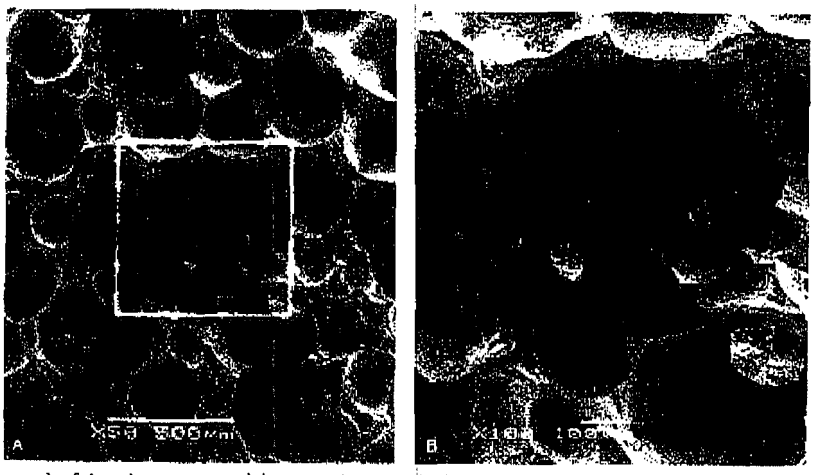

Fig. 1. (A) SEM photomicrograph of the microstructure of the synthetic porous hydroxyapatite. Bar = 500 μm. (B) Higher magnification of the rectangular area in A. Diameter in interconnecting path was 200 μm. Bar = 100 μm.

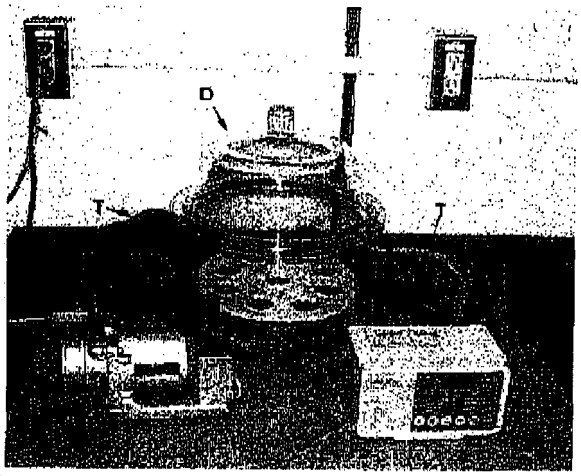

Fig. 2. Photograph of low-pressure system. V indicates Ulvac G-5 oil rotary vacuum pump; C indicates Iuchi vacuum controller VC-100; D indicates Iuchi polycarbonate vacuum desiccator; T indicates rubber tube and silicon tube.

Hg as an example. First, we chose Program Mode and set 100 mm Hg as the Pressure Value, with 10% Upper Limit and 150 mm Hg Slope Point. After setting the vacuum controller, the culture dish was put into the polycarbonate vacuum desiccator, which was then closed. The vacuum pump was turned on and the vacuum controller was started by pushing the Start button on the front panel and finished by pushing the End button. The whole application time of low pressure was 100 s. Experiments with other pressure groups were preformed similarly.

We examined six groups (group 1 to group 6) with different pressures; 760 (normal atmospheric pressure), 500, 250, 100, 50, and 10 mm Hg.

After treatment with the low-pressure system, the culture dish was incubated for 2 h at 37 °C at normal atmospheric pressure. Then, each HA/BMO composite was transferred to a new 24-well plate (Falcon). Each HA block was subcultured in one well with 2 ml of osteogenic medium. The osteogenic medium consisted of standard medium supplemented with 50 μg/ml of vitamin C phosphate (L-ascorbic acid phosphate magnesium salt *n*-hydrate, Wako, Osaka, Japan), 10 mM Na β-glycerophosphate (Merck Japan, Tokyo, Japan), and 10^{-8} M dexamethasone (Dex, Sigma). The medium was renewed every other day and the subcultures were maintained for 2 weeks [2,3]. Then, HA/BMO composites were implanted subcutaneously sites in rats as described below.

2.3. Surgical procedure

Syngeneic 7 week-old male Fischer rats were anesthetized by intramuscular injection of pentobarbital (Nembutal 3.5 mg/100 g BW), following light ether inhalation. Six subcutaneous pouches were created in the back of the rat following small incisions made for ceramic implantation. Six HA/BMO composites from six groups after 2 weeks of subculture were implanted subcutaneously at six sites on the back of each syngeneic rat.

A total of 240 porous HA blocks were used in the present study. Twelve HA/BMO blocks, obtained from different rats, were used for biochemical analysis and 12 HA/BMO blocks were used for histological analysis at each time point.

2.4. Biochemical analysis

The composites were harvested and used for biochemical analysis at 4 and 8 weeks (12 HA/BMO blocks each) after implantation. Alkaline phosphatase (ALP) activity and bone osteocalcin (OCN) content of the harvested HA blocks were determined [7]. Briefly, implants were immediately crushed with a hammer, homogenized in 0.5 ml of 0.2% Nonidet P40 containing 1 mM $MgCl_2$ with Physcotron (Micro-Tech Niti-on, Funahashi City, Chiba, Japan), and centrifuged at 13,000 rpm for 10 min at 4 °C. The supernatant was assayed for ALP activity using *p*-nitrophenylphosphate as the substrate. An aliquot (2.5 μl) of supernatant was added to 0.5 ml of 50 mM *p*-nitrophenylphosphate containing 1 mM $MgCl_2$ and the mixture was incubated for 30 min at 37 °C. Then, 0.5 ml of 0.2 N NaOH was added to stop the reaction, and the absorption at 410 nm was measured with a spectrophotometer. ALP activity was determined as millimolars of *p*-nitrophenol released per implant after 30 min of incubation at 37 °C.

OCN was extracted from the sediment after extraction using 0.2% Nonidet P40 by shaking in 5 ml of 20% formic acid for 1 week at 4 °C. An aliquot (2 ml) of the formic acid extract was then applied to a column of Sephadex G-25 (PD-10) and eluted with 10% formic acid. Protein fractions were collected, lyophilized, and prepared for the assay of intact rat OCN as previously described [6]. The method utilized two antibodies that recognized the N- and C-terminal amino acid regions of OCN.

All assays were performed with rabbit antiserum to rat OCN and purified rat OCN as a standard and tracer. We used a Rat Osteocalcin EIA Kit (No.BT-490 Biomedical Technologies, USA) and followed the manufacturer's protocol.

2.5. Statistical analysis

Average values of ALP activity and OCN content were expressed as the arithmetic mean ± SE. Analysis of data was performed by paired *t*-test. Differences were considered statistically significant when the P value was < 0.01 or 0.001.

2.6. Histological analysis

Half of the composites were harvested and used for histological analysis by SEM at 2 and 4 weeks (12

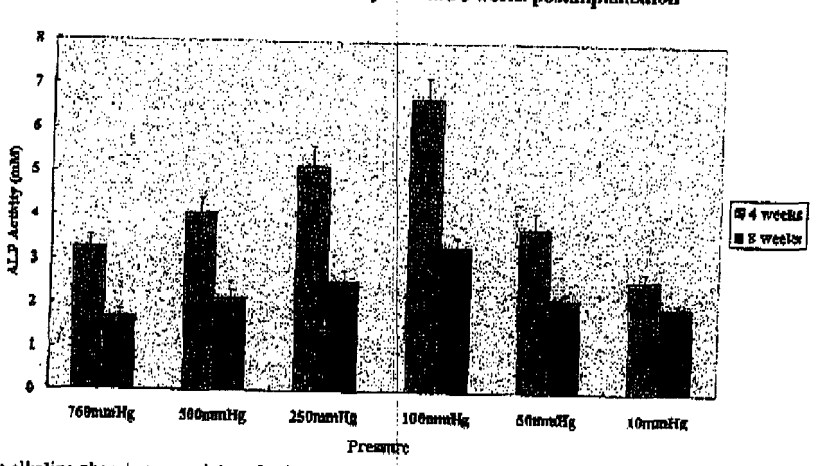

Fig. 3. Temporal changes in alkaline phosphatase activity of subcultured composite grafts at 4 and 8 weeks post-implantation. The data are the mean ± SE ($n = 12$). Significant differences compared with values of the 760-mm Hg group. * $P < 0.01$ by paired t-test analysis, ** $P < 0.001$. Details are described in Materials and methods.

HA/BMO blocks each) after implantation, and the other half were observed by light microscopy at 4 and 8 weeks (12 HA/BMO blocks each) after implantation.

A series of samples of BMO/HA composites were prepared for scanning electron microscopic (SEM) analyses. The composites were evenly cut at the central part of the HA block. Then, specimens were fixed with 2% paraformaldehyde/2.5% glutaraldehyde in 0.1 M Na cacodylate buffer (pH 7.4), post-fixed with 0.1% osmium tetroxide, dehydrated in a graded alcohol series, and dried in a critical point dryer. The composites were fixed onto scanning electron microscopy stubs with tape, sputter-coated with platinum/gold in an ion coater, and observed with an SEM (Hitachi S-4500, Japan).

For light microscopic observation, the composites were fixed in 10% buffered formalin, decalcified (K-CX solution, Falma, Tokyo, Japan), embedded in histparaffin, and stained with hematoxylin and eosin. These specimens were observed under an optical microscope (Leica Dmr, Germany).

3. Results

3.1. Biochemical findings

A comparison of ALP activity in subcultured HA/BMO composite grafts in different pressure groups at 4 and 8

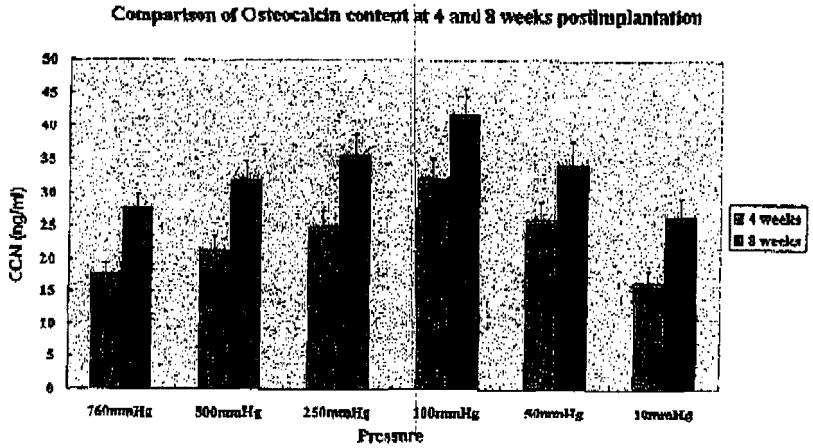

Fig. 4. Temporal changes in osteocalcin content of subcultured composite grafts at 4 and 8 weeks post-implantation. The data are the mean ± SE ($n = 12$). Significant differences are compared with values of the 760-mm Hg group. * $P < 0.01$ by paired t-test analysis, ** $P < 0.001$. Details are described in Materials and methods.

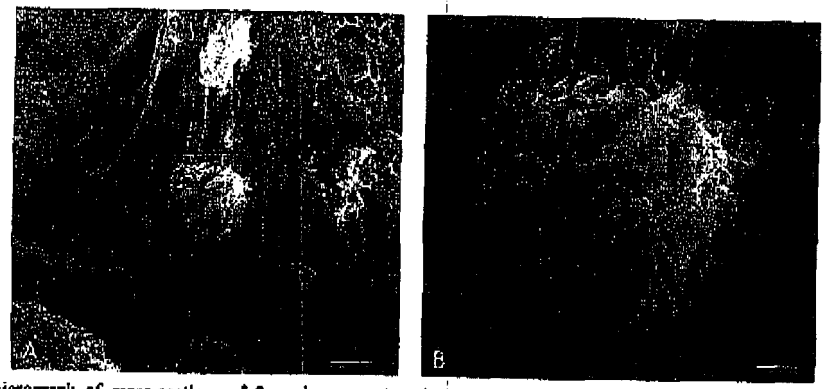

Fig. 5. (A) SEM photomicrograph of cross-sections of 2-week composite after implantation. The pore surface is covered by round cells as well as collagenous extracellular matrix. E indicates collagenous extracellular matrix. Bar = 37.5 μm. (B) Higher magnification of the large rectangular area in A. Round cell, which seems to be active osteoblast, can be seen on the surface of HA. Bar = 5 μm.

week post-implantation is shown in Fig. 3. The ALP activity in HA/BMO composite subjected to a pressure of 100 mm Hg was the highest among the six groups at 4 or 8 weeks after implantation. The level of ALP activity increased until pressure decreased to 100 m Hg. Then, the level of activity decreased again with subsequent pressure decreases. Both ALP activities at 4 and 8 weeks post-implantation showed similar expression, but the level of activity after 8 weeks were lower than that after 4 weeks.

A comparison of bone OCN content in subcultured HA/BMO composite grafts at 4 and 8 weeks post-implantation is shown in Fig. 4. The bone OCN content in HA/BMO composite treated at a pressure of 100 mm Hg was also the highest among six groups at 4 or 8 weeks after implantation. Both OCN levels at 4 and 8 weeks post-implantation showed maximum levels at 100 mm Hg. The level of activity increased until pressure decreased to 100 m Hg. Then, the level of activity decreased again with pressure decreases. However, the level of OCN after 8 weeks was higher than that after 4 weeks.

3.2. Histological findings

SEM analysis of the 2-week subcultured HA/BMO composites treated at 100 mm Hg suggested that most of the pore surface was covered by round cells together with collagenous or non-collagenous extracellular matrices as shown in Fig. 5.

At 4 weeks after implantation, light microscopic images of decalcified section with HE staining showed mature bone areas and the number of active osteoblasts facing the bone increased in pores (Fig. 6). All HA/BMO composites treated at 100 mm Hg showed bone formation in the

Fig. 6. Subcultured composite 4 weeks after implantation. Hematoxylin and eosin stain; white area shows the ghost of hydroxyapatite ceramic (H) produced by decalcification. B indicates the more mature bone tissue in the porous area; C indicates mature osteocyte; O indicates active osteoblast forming bone; V indicates vasculature; I indicates interconnecting path. Original magnification ×200.

Fig. 7. Subcultured composite 8 weeks after implantation. Hematoxylin and eosin stain; the bone was more abundant in the porous area. Original magnification ×400. B indicates mature bone tissue; C indicates osteocyte; P indicates pore.

pore regions. Small round cells were rarely seen in the pore regions, indicating a minimal inflammatory response. However, not all of the composites in the normal pressure or other pressure groups showed bone formation. A palisade-like arrangement of cuboidal active osteoblasts was seen in many of the pore areas, and thus the bone formation was active and progressive. At 8 weeks, the quantity of bone was greater than that at 4 weeks (Fig. 7).

4. Discussion

The bone marrow stromal system is currently thought to be the reservoir of bone precursor cells. In particular, the mesenchymal component of bone marrow cells (BMO) can support the self-repair of bone tissue because it contains a low but extremely active fraction of multipotent precursor [8].

With the culture conditions described in the experimental section, after 2 weeks of subculture, the BMOs readily differentiated into osteoblasts and the osteoblasts fabricated mineralized bone matrix on the pore surface of HA [2,9].

ALP activity reflects the osteoblastic activity, and bone osteocalcin content is correlated well with the amount of newly formed bone in the composites of HA/BMO after subcutaneous implantation [7]. Both ALP activity and OCN content are useful as biochemical markers of osteogenesis [10–12]. Therefore, osteoinduction can be marked by increased ALP expression and the formation of colonies competent in mineralization. During the induction period, when cells organize into nodules and mineralize, the OCN content is significantly elevated along with the onset of extracellular matrix mineralization [13].

The present study showed that the ALP activity did not increase monotonously when the pressure decreased. We considered it necessary for osteoblasts to grow in the presence of a definite oxygen concentration. An appropriate low oxygen concentration can stimulate osteoblast differentiation [14]. Increased vascular endothelial growth factor expression in osteoblasts can be induced by hypoxia. In this way, hypoxia results in increased ALP activity [15]. Numerous studies have demonstrated the critical role of angiogenesis for successful osteogenesis during endochondral ossification and fracture repair. Hypoxia can also induce insulin-like growth factors (IGF) I and II—cytokines that were believed to play a role in increased collagen synthesis. However, oxygen concentrations that were too low also injured osteoblasts and decreased the ALP activity [16].

The 10-mm Hg program, with an oxygen pressure of only 2 mm Hg, would last for more than 3 min. We considered pressures less than 100 mm Hg to be harmful for osteoblast differentiation.

Appropriate low pressure here had two possible effects: removal of air within the pores of the ceramic facilitating the flow of cell suspension into the pores; and induction of some growth factor(s), which had a significant role in osteoblast differentiation. We speculated that hypoxia may have been responsible for the effects seen in the present study, although we have no direct evidence for this. Therefore, further studies are required to resolve this issue.

By light microscopy, we found many active osteoblasts on the surface of newly formed bone tissue. Scanning electron microscopy revealed many collagenous extracellular matrices with osteoblasts on the surface of pores. These findings indicated that the pressure chosen here was appropriate for osteoblasts to differentiate into osteocytes.

Bruder et al. [17] used a vacuum in tissue engineering research, but they did not clarify or analyze the pressure dependence of in vivo bone formation in detail. The present study clearly demonstrated that the amount of bone formation in vivo is strongly correlated with applied pressure when HA blocks are soaked in cell suspension.

In conclusion, applying an appropriate low pressure when HA blocks are soaked in cell suspension is of benefit to increase bone tissue formation.

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